

ORIGINAL RESEARCH



Inflammation drives nitric oxide synthase 2 expression by $\gamma\delta$ T cells and affects the balance between melanoma and vitiligo associated melanoma

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ABSTRACT

The high expression of inducible nitric oxide synthase (NOS2) by myeloid-derived suppressor cells (MDSCs) is a key mechanism of immune evasion in cancer. Recently we reported that NOS2 is also expressed by $\gamma\delta$ T cells in melanoma, contributing to their polarization towards a pro-tumor phenotype. The molecular mechanisms underlying regulation of NOS2 expression in tumor-induced $\gamma\delta$ T cells remain unexplored. By using the model of mice transgenic for the ret oncogene (Ret mice) that develops a spontaneous metastatic melanoma, we evidence that interleukin (IL)-1 β and IL-6 drive NOS2 expression in $\gamma\delta$ T cells. Indeed, their *in vivo* neutralization lessens the $\gamma\delta$ T cell capacity to produce not only NOS2, but also IL-17 involved in the recruitment of MDSCs at the primary tumor site. The treatment also delayed tumor cell dissemination and induced vitiligo in a significant proportion of Ret mice. Interestingly, Ret mice developing a less aggressive melanoma, characterized by the spontaneous development of a concomitant autoimmune vitiligo, exhibit a weaker concentration of inflammatory cytokines and a reduction of tumor infiltrating $\gamma\delta$ T cells expressing NOS2, when compared to Ret mice without any signs of vitiligo. Overall our results support that the level of inflammation at the tumor site regulates NOS2 expression by $\gamma\delta$ T cells and the development of vitiligo associated melanoma.

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Introduction

Melanoma represents 5% of skin cancer and is the deadliest form due to its high metastatic potential. Metastasis formation is a multistep process starting by tumor cell dissemination, an event that occurs early, even before the clinical diagnosis of the primary tumor.¹ Metastasis is not only directed by cancer-cell-intrinsic mechanisms that allow tumor cells to invade the local microenvironment, reach the circulation, and colonize distant sites but also by the tumor microenvironment within myeloid derived suppressor cells (MDSCs) play a key role.

Polymorphonuclear (PMN-) MDSCs are the major subset of MDSCs that accumulates at the primary tumor site.² These cells favor tumor progression by inhibiting anti-tumor responses, by inducing angiogenesis or by initiating a pre-metastatic niche.³ Furthermore, tumor infiltrating PMN-MDSCs contribute to cancer cell dissemination via epithelial-mesenchymal transition of melanoma cells.⁴ Many reports indicated that these cells are recruited by gamma delta ($\gamma\delta$) T cells.

The cellular and cytokine composition of the tumor microenvironment can affect the $\gamma\delta$ T cell plasticity and subsequently their anti-tumor properties.⁵ This may explain in part the dual role of these T cells in both human cancers and mouse models.⁶ Indeed, protective role of $\gamma\delta$ T cells has been established in mouse models (reviewed in ⁷). A meta-analysis evidenced an

association between their proportion within the microenvironment in a multitude of human malignancies and a favorable prognosis.⁸ However, $\gamma\delta$ T cells can also promote tumor development and metastasis, for instance via expressing PDL1 and galectin 9⁹ or producing IL-17 in both human and rodent cancers.^{10–12} Recently, we identified nitric oxide synthase 2 (NOS2) that catalyzes the production of nitric oxide (NO) and L-citrulline from L-arginine, as a major driver of a pro-tumor profile in $\gamma\delta$ T cells by favoring their IL-17 production.¹³ Indeed, NOS2 leads to the recruitment of PMN-MDSCs at the primary tumor site and subsequently to metastasis formation in melanoma. In this model, mice are transgenic for the activated *ret* oncogene (Ret mice) and develop a metastatic melanoma.^{14,15} A spontaneous vitiligo occurs in nearly forty percent of cases resulting in a delayed incidence of cutaneous and distant metastasis.¹⁵

Melanoma patients developing a vitiligo-like depigmentation, either spontaneously or in response to immunotherapy¹⁶ are partially protected against tumor progression. This autoimmune skin disease is associated with more favourable clinical outcome.^{17,18} Vitiligo results from a robust anti-melanoma immunity mainly mediated by CD8⁺ T cells that also recognize normal melanocytes sharing expression of melanocyte differentiation antigens.^{19–21}

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In the present study, we pursued the investigation that auto-crine NOS2 expression in tumor infiltrating $\gamma\delta$ T cells plays a role in the tumor aggressiveness. By using the Ret model, we further show that IL-1 β and IL-6 promote NOS2 expression in $\gamma\delta$ T cells and control the balance between melanoma and vitiligo associated melanoma.

Results

Vitiligo development delays melanoma progression less efficiently than genetic *Nos2* inactivation

Vitiligo occurs spontaneously in 35 to 40% of Ret mice and the metastatic melanoma progression is significantly delayed in mice exhibiting vitiligo (RetV) compared to mice with normal skin (RetNV) (¹⁵, and Figure 1). Recently, we have shown a delay of tumor onset in Ret mice deficient for NOS2 (RetNos2KO).¹³ Interestingly we show that the protection of these mice is not due to a higher incidence of vitiligo because only 35% of 6 month old RetNos2KO mice display skin depigmentation (Figure 1A). Regarding the impact on tumor outgrowth, the kinetics of primary tumor incidence are similar in RetV and RetNos2KO mice (Figure 1B), but the spontaneous vitiligo development is less efficient than the genetic *Nos2* inactivation to limit metastasis formation (Figure 1C). These results suggest the involvement of NOS2 in the vitiligo development.

Vitiligo development is associated with a decrease of PMN-MDSCs and $\gamma\delta$ T cells in primary tumors

To decipher whether the tumor protection associated to vitiligo relies on a specific immune microenvironment at the primary tumor site, we analyzed *ex vivo* the cytokine profile of primary tumors derived from 6 month old RetV and RetNV mice. The protein levels of IL-12p70, IFN- γ and tumor necrosis factor- α (TNF- α) were quite similar in both groups (Figure 2A). Primary tumors from RetV mice contained lesser amounts of IL-10 and keratinocyte-derived cytokine (KC), a murine IL-8 homologue involved in PMN cell recruitment, than RetNV mice (Figure 2A). Interestingly, they also displayed less IL-1 β and IL-6 (Figure 2A). Although

circulating IL-1 β was almost undetectable in both groups, the concentration of IL-6 was lower in the blood of RetV mice than in RetNV mice (Figure 2B), consistent with our data from aqueous humors (primary tumor site). This result reinforces that, in presence of vitiligo onset, inflammation is significantly reduced in the course of the melanoma development.

In parallel, we quantified the immune cells within primary tumors of 6 month old mice (Figure 2C-F). Primary tumors of RetV mice are more infiltrated by anti-tumor effector cells, such as B cells, NK cells and tumor infiltrating lymphocytes (TILs), than those from RetNV mice (Figure 2D). Remarkably they also exhibited significantly less PMN-MDSCs (CD11b⁺CD11c⁻Ly6C^{low}Ly6G⁺, mean: 36.8% vs 9.8%) and $\gamma\delta$ T cells (CD3⁺, pan $\gamma\delta$ TCR, mean: 5.1% vs 3%) (Figure 2E), consistent with the low levels of KC and with the key roles of these immune subsets in the Ret melanoma progression.^{4,13} Similarly PMN-MDSCs and $\gamma\delta$ T cells were less frequent in the spleen of RetV mice than of RetNV mice (Figure 2F). Collectively, our results suggest that the reduced proportion of $\gamma\delta$ T cells at the primary tumor site and the subsequent reduced proportion of PMN-MDSCs contribute to delay the tumor development in Ret mice with melanoma-associated vitiligo.

Vitiligo development is associated with a decrease of $\gamma\delta$ T cells expressing NOS2

Our recent data evidenced that NOS2 expressing $\gamma\delta$ T cells lead to a strong recruitment of PMN-MDSCs.¹³ Because RetV and RetNos2KO mice display the same delay in primary tumor onset, we hypothesized that $\gamma\delta$ T cells in RetV mice express a lower level of NOS2 than RetNV mice. In this aim, we isolated CD45⁺ cells from primary tumors and tumor draining lymph nodes (LN), and stained them for NOS2 in green and TCR δ chain in red. The Figure 3A illustrates a representative staining from LN derived cells. After undergoing the cytopspin process, $\gamma\delta$ T cells producing or not NOS2 were quantified. Interestingly, we found that the NOS2 expressing $\gamma\delta$ T cells among all $\gamma\delta$ T cells are significantly less frequent in LN (Figure 3B) as well as

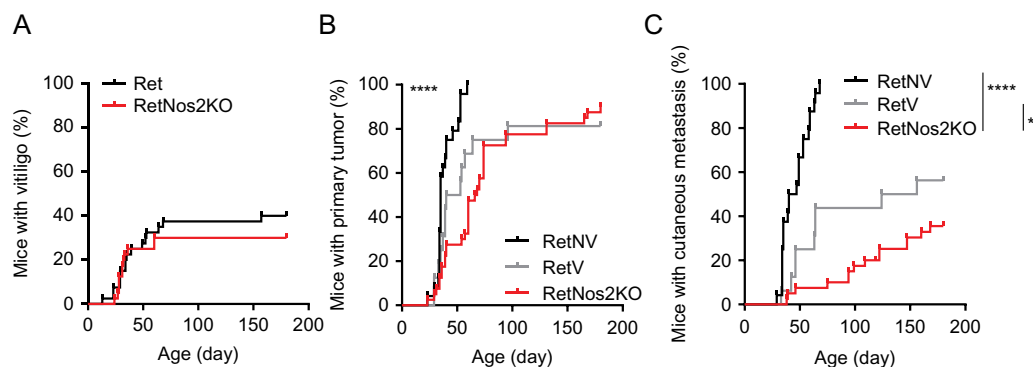


Figure 1. Vitiligo confers an intermediate protection against melanoma development compared to NOS2 deficiency.

(A) Time courses of vitiligo onset from Ret (n = 40) and RetNos2KO (n = 40) mice. (B-C) 6-months follow-up of melanoma development from Ret mice without vitiligo (RetNV, n = 24), Ret mice with vitiligo (RetV, n = 16) and RetNos2KO (n = 40) mice. Time courses of primary tumor (B) and cutaneous metastasis (C) onset. Mice were examined every two weeks. **P* < 0.05 and *****P* < 0.0001 (Wilcoxon test).

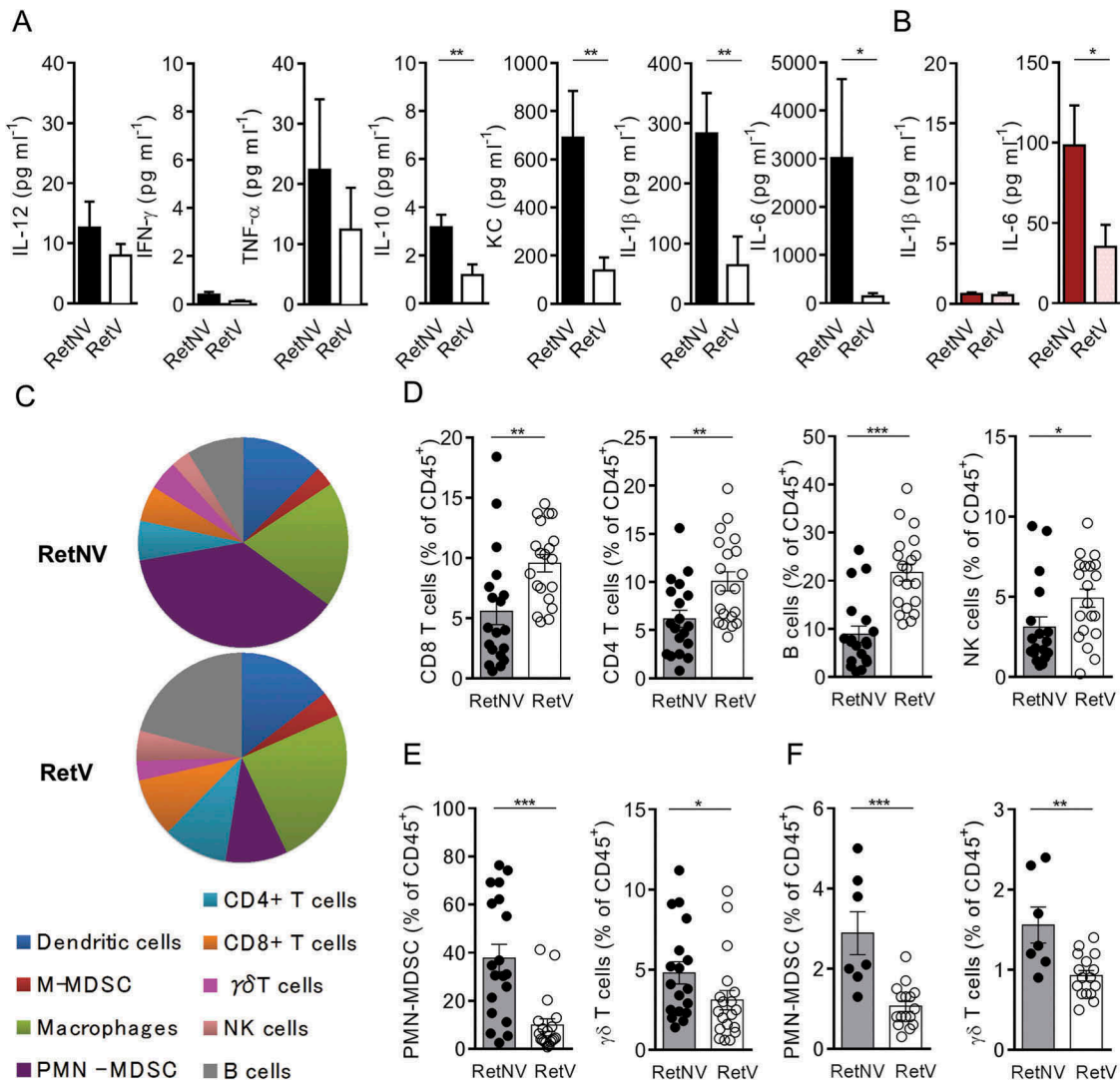


Figure 2. Vitiligo-associated decrease of PMN-MDSCs and $\gamma\delta$ T cell infiltration within primary tumors and spleen.

(A) Protein levels of indicated cytokines in aqueous humors from RetNV ($n = 14$) and RetV ($n = 11$) mice, determined by multiplex ELISA. (B) IL-1 β and IL-6 quantifications in sera from RetNV ($n = 8$) and RetV ($n = 8$) mice determined by ELISA. (C-F) Dendritic cells (CD11c⁺), macrophages (CD11b⁺CD11c⁺Ly6C^{low}Ly6G⁻), monocytic MDSCs (M-MDSCs: CD11b⁺CD11c⁺Ly6C^{high}Ly6G⁻), PMN-MDSCs (CD11b⁺CD11c⁺Ly6C^{low}Ly6G⁺), CD4⁺ T cells (CD3⁺, CD4⁺), CD8⁺ T cells (CD3⁺, CD8⁺), $\gamma\delta$ T cells (CD3⁺, δ TCR⁺), NK cells (CD3⁺, NK1.1⁺), B cells (CD3⁺, CD19⁺) were analyzed by flow cytometry. Parts of whole graphs describing immune cells found in primary tumors (C). Percentages of CD8⁺ T cells, CD4⁺ T cells, NK cells and B lymphocytes (D) PMN-MDSCs and $\gamma\delta$ T cells among CD45⁺ cells within primary tumors (E) from RetNV ($n = 18$) and RetV ($n = 19$) or within spleen (F) from RetNV ($n = 7$) and RetV ($n = 16$). Each point represents individual mouse. Bars are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ (Mann-Whitney test).

within the primary tumor (Figure 3C) of RetV mice compared to RetNV mice. Our results indicate that vitiligo occurrence is associated with a decrease of $\gamma\delta$ T cells producing NOS2.

In vivo neutralization of IL-1 β and IL-6 slows down metastasis formation, induces vitiligo incidence and lessens recruitment of $\gamma\delta$ T cells and PMN-MDSCs

As weak amounts of IL-1 β and IL-6 were detected in primary tumor extracts from RetV mice (Figure 2A), we assumed that these cytokines play a key role in inducing NOS2 in $\gamma\delta$ T cells that could subsequently accelerate tumor onset. To address this point, Ret mice were treated either with antibodies neutralizing IL-1 β and IL-6 or with the IL-1 receptor antagonist anakinra during three months (Figure 4). These treatments did not affect primary

tumor incidence (Figure 4A), but significantly delayed metastasis formation (Figure 4B). IL-1 β and IL-6 neutralization tends to control metastasis dissemination more efficiently than pharmaceutical inhibition of the IL-1 receptor (Figure 4B). The frequency of vitiligo was similar in Ret mice either untreated (38.6%, Figure 1A) or treated with anakinra (35.6%, Figure 4C) at the end of the follow-up period. Among the twelve Ret mice used to neutralize IL-1 β and IL-6, four mice (33.3%) exhibited vitiligo before treatment. Interestingly, four RetNV mice became depigmented during treatment. The comparison of the occurrence of vitiligo between treated mice (66.7%, Figure 4C) and untreated mice (Figure 1A) suggests that cytokine neutralization induced the development of vitiligo. Nor IL-1 β or IL-6 amounts were found in aqueous humors of 3 month treated mice confirming the neutralization efficiency (Figure 4D). As shown by cytometry, the neutralization of IL-1 β and IL-6 significantly reduced the proportion

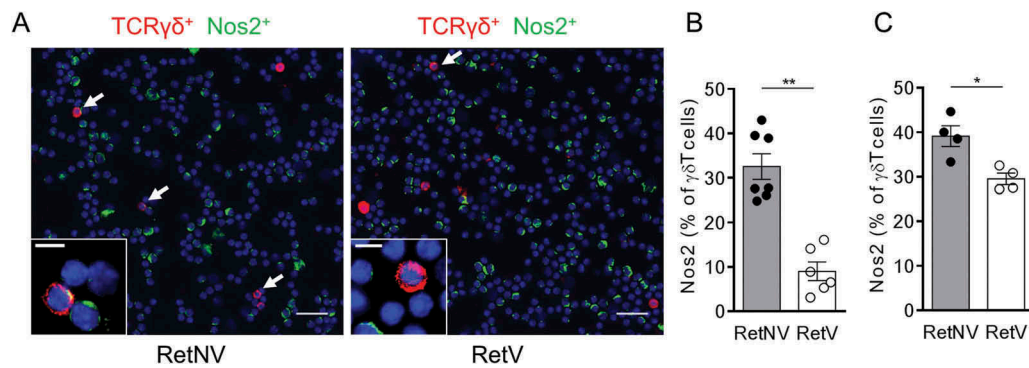


Figure 3. Vitiligo-associated decrease of the proportion of NOS2 positive $\gamma\delta$ T cells in primary melanoma and TdLNs.

(A) Representative microscopy images showing $\gamma\delta$ T cells positive for NOS2 from cells derived from TdLNs of RetNV or RetV mice and stained with antibodies to TCR $\gamma\delta$ (red), NOS2 (green) and counterstained with DAPI (blue). Arrows indicate NOS2 expressing $\gamma\delta$ T cells. Bars 10 μ M. 40 X objective. (B, C) Quantification of NOS2 positive $\gamma\delta$ T cells in TdLN (B) from RetNV ($n = 7$) and RetV ($n = 6$) and in primary melanoma (C) from RetNV and RetV ($n = 4$ each). It was performed from 500 to 1500 $\gamma\delta$ T cells. Each point represents individual mouse. Bars are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ (Mann-Whitney test).

of PMN-MDSCs and $\gamma\delta$ T cells within primary tumors (Figure 4E, F) and spleen (Figure 4G, H), consistent with the effect on the metastatic control. Taken together, these data showed that IL-1 β and IL-6 promote metastasis formation via an effect on PMN-MDSCs and $\gamma\delta$ T cells in our melanoma model.

In vivo neutralization of IL-1 β and IL-6 reduces NOS2 expression in pro-tumorigenic $\gamma\delta$ T cells

We next evaluated if IL-1 β and IL-6 contribute to NOS2 expression in $\gamma\delta$ T cells. Three month old RetNV mice were treated with anti-IL-1 β and anti-IL-6 neutralizing antibodies. After fifteen days of treatment, the proportion of NOS2 expressing $\gamma\delta$ T cells, assessed by microscopy, was significantly reduced (Figure 5A and B). These cells represented less than 10% of $\gamma\delta$ T cells in treated RetNV mice (Figure 5B), a proportion quite similar to that observed in untreated RetV mice (Figure 3B). Interestingly, we also found lower amounts of IL-17 in primary tumors extracts from treated RetNV mice (Figure 5C), suggesting that such a short-term treatment already affects the proportion of IL-17 producing $\gamma\delta$ T cells at the tumor site. Collectively, our results showed that high concentrations of IL-1 β and IL-6 within the tumor microenvironment strongly supported NOS2 expression in pro-tumorigenic $\gamma\delta$ T cells in melanoma and that their low NOS2 expression may, at least in part, explain the reduced tumor aggressiveness in Ret mice with melanoma-associated vitiligo.

Discussion

Here, we demonstrated that IL-1 β and IL-6 inflammatory cytokines induce NOS2 expression by $\gamma\delta$ T cells in a spontaneous model of melanoma. We previously reported that NOS2 drives optimal IL-2 and IL-17 production, proliferation and glycolysis of $\gamma\delta$ T cells promoting their peripheral expansion at steady state and their pro-tumoral properties in the context of melanoma,^{13,22} nevertheless the molecular mechanisms underlying autocrine NOS2 expression by $\gamma\delta$ T cells were not investigated. Murine interferons (IFN- γ and type I IFNs) and microbial products (e.g., LPS/TLR ligand activated

NF- κ B) up-regulate NOS2 in myeloid cells, particularly in macrophages.²³ IL-1 β and/or IL-6 induce NOS2 expression in several immune populations including macrophages, plasma cells and Th17 cells.^{24–27} Recently, the SETD1B histone methyltransferase was shown to activate NOS2 in tumor-induced MDSCs.²⁸ Indeed, the regulation of NOS2 is different depending on immune cell types. Here, firstly we observed that the primary tumor in Ret mice with the more aggressive melanoma is particularly enriched in IL-1 β and IL-6 and highly infiltrated within NOS2 expressing $\gamma\delta$ T cells and secondly that IL-1 β and IL-6 neutralization reduced the capacity of $\gamma\delta$ T cells to express NOS2 and their proportion at the tumor site. Collectively, our data support a key role of these inflammatory cytokines in regulating NOS2 expression by $\gamma\delta$ T cells *in vivo*. Moreover we have previously observed that IL-1 β and IL-6 were almost absent from primary tumor extracts of RetNos2KO mice¹³ suggesting a possible feedback loop between NOS2 and these cytokines. Such an hypothesis is supported by the induction of IL-6 transcription by nitric oxide via activating guanosine 3',5'-cyclic monophosphate-dependent protein kinase in osteoblasts and skeletal myotubes.^{29,30}

Melanoma patients may develop spontaneously a vitiligo that correlates with significant improved 5-year survival.^{17,18,31} When occurring in response to melanoma treatment (i.e. anti-PD1 and anti-CTL-A4 based immunotherapy), the occurrence of vitiligo may also have prognostic significance.²⁰ An autoimmune etiology has been proposed to explain the development of vitiligo essentially based on the presence of autoantibodies and autoreactive T lymphocytes against melanocyte antigens.³² Nevertheless, an effort has to be done to identify new immune-related actors involved in the balance between autoimmunity and melanoma to provide new insight into the development of more efficient melanoma treatment. Using the Ret mouse model, we have previously reported that mice developing a melanoma-associated vitiligo displayed frequent peripheral melanoma-specific CD8⁺ T-cells that protected them toward a challenge with syngenic melanoma cells.¹⁵ In line with this, CD8⁺ T cells specific for melanocyte-differentiation antigens are detected in the human related pathology.²¹ Despite our current observation that tumor infiltrating CD8⁺ T cells are more frequent in

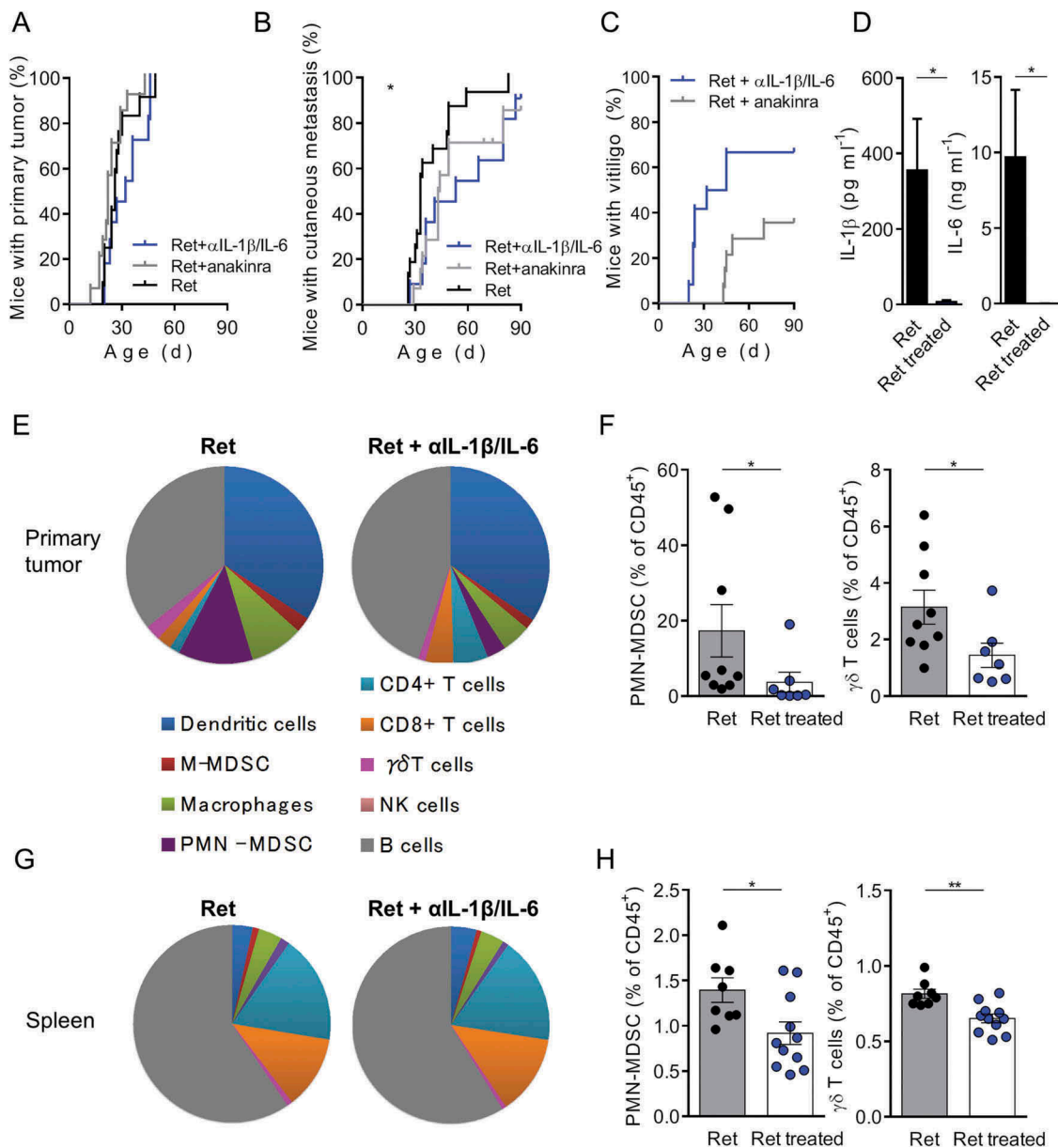


Figure 4. *In vivo* neutralization of IL-1 β and IL-6 slows down metastasis formation and reduced PMN-MDSCs and $\gamma\delta$ T cell infiltration within primary tumors and spleen.

(A, B) 3-month follow-up of melanoma development from Ret mice untreated ($n = 12$) or treated either with antibodies neutralizing IL-1 β and IL-6 ($n = 12$) or with IL-1 receptor antagonist (anakinra) ($n = 14$). Time courses of primary tumor (A), cutaneous metastasis (B) and vitiligo (C) onsets. (D) Protein levels of IL-1 β and IL-6 in primary tumor extracts, from Ret ($n = 4$) and Ret treated ($n = 4$) mice, determined by multiplex ELISA. (E-H) Analyses of immune cell proportions within primary tumors (E) and spleen (G) represented as parts of whole. Percentages of PMN-MDSCs and $\gamma\delta$ T cells among CD45⁺ cells within primary tumors (F) or spleen (H) from Ret ($n = 9$ and 8) and Ret treated ($n = 7$ and 11 respectively) mice. Each point represents individual mouse. Bars are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$. Wilcoxon test (A, B), Mann-Whitney test (D, F, H).

RetV mice compared to RetNV mice, Ret mice either deficient for T cells or depleted for CD8⁺ T cells from birth developed a melanoma-associated vitiligo,^{33,34} supporting that CD8⁺ T cells would be the consequence of the tumor immune surveillance rather than the cause of the vitiligo development. We also evidenced accumulation of inflammatory monocytes/dendritic cells inhibiting tumor cell proliferation in the skin of Ret mice with active vitiligo and demonstrated their key contribution in the tumor control resulting in skin depigmentation.^{34,35} Here, we analyzed for the first time the cytokine profile and the composition of immune cells infiltrating the primary tumor in Ret mice with or without vitiligo. In addition to CD8⁺ T cells

already discussed, CD4⁺ T, B and NK cells infiltrate more significantly tumors of RetV mice compared to those of RetNV mice. We have shown that T, B and NK cells are not crucial for the development of vitiligo in Ret mice and that IL-10 neutralization results in increased occurrence of vitiligo³⁴ consistent with the reduced IL-10 expression in RetV mice compared to RetNV mice we observed here. Strikingly, NOS2 expressing $\gamma\delta$ T cells and PMN-MDSCs are less frequent in mice developing a spontaneous melanoma associated vitiligo, in agreement with the reduced local concentration of IL-1 β , IL-6 and KC. In line with these data, we recently found that NOS2 promoted IL-17 production by tumor infiltrating $\gamma\delta$ T cells leading to the

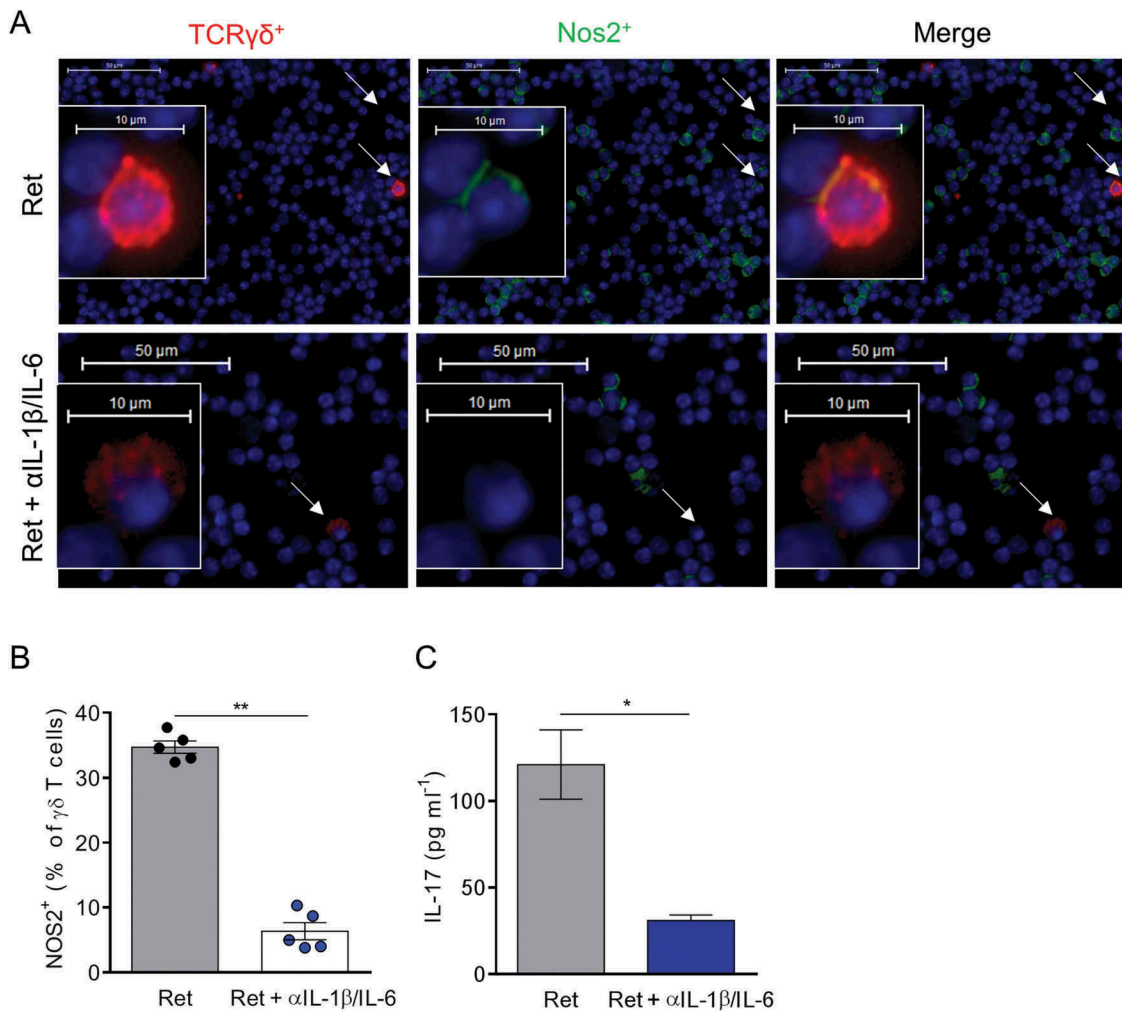


Figure 5. *In vivo* neutralization of IL-1 β and IL-6 suppresses NOS2 expression in $\gamma\delta$ T cells.

(A–C) 3 month old Ret mice were treated or not during 2 weeks with antibodies neutralizing IL-1 β and IL-6. (A, B) NOS2 expression was analyzed by microscopy in TdLNs from 5 mice in each group. Representative images showing $\gamma\delta$ T cells positive for NOS2 derived either from untreated or treated Ret mice and stained with antibodies to TCR $\gamma\delta$ (red), NOS2 (green) and counterstained with DAPI (blue). Bars 10 μ m. 40 X objective. Arrows indicate $\gamma\delta$ T cells. (B) NOS2 positive $\gamma\delta$ T cells were quantified from 500 to 1500 $\gamma\delta$ T cells. (C) Protein levels of IL-17 in primary tumor extracts, from untreated ($n = 5$) and treated ($n = 5$) Ret mice, were determined by ELISA. Each point represents individual mouse. Bars are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$. (Mann-Whitney test).

recruitment of PMN-MDSCs.¹³ In addition, the neutralization of IL-1 β and IL-6 reduced NOS2 expression in $\gamma\delta$ T cells. Collectively, our data suggest that the delay in tumor cell spreading in melanoma associated vitiligo is due, at least in part, to a reduced presence of IL-1 β and IL-6 at the primary tumor site, decreasing the proportion of NOS2 expressing $\gamma\delta$ T cells and subsequently the recruitment of PMN-MDSCs.

Chronic inflammation in the tumor microenvironment is an hallmark of cancer that promotes tumorigenesis and tumor cell dissemination.^{36,37} Counteracting cancer induced chronic inflammation may be an attractive therapeutic strategy,³⁸ as exemplified with the reduced B16 melanoma cell tumorigenicity in IL-1 β knockout mice³⁹ or the lower incidence of lung cancer incidence and mortality after IL-1 β inhibition.⁴⁰ Here, we show not only a delay in tumor cell dissemination, but also a significant increase of the occurrence of vitiligo in Ret mice in response to IL-1 β and IL-6 neutralization, suggesting a key role of inflammatory cytokines in the balance between cancer and autoimmunity.

In conclusion, our data show for the first time that the levels of IL-1 β and IL-6 at the tumor site control the recruitment of $\gamma\delta$ T cells producing NOS2 and the balance between melanoma and vitiligo associated melanoma.

Materials and methods

Mice

MT/ret $^{\pm}$ mice (called Ret later) are transgenic for the human Ret oncogene.¹⁴ Ret mice were crossed with mice deficient for Nos2 to obtain RetNos2KO mice. The incidences of primary tumors and cutaneous lesions were assessed from day 21 and then twice a month. 3 to 6-month-old mice were used for experiments. Mice were sacrificed at indicated times or when considered moribund (prostrated, bristly, skinny). All mice are on the C57BL/6J background and were maintained in our animal facility under specific pathogen-free conditions.

Protein quantifications at the primary tumor site and in sera

Aqueous humors of 6 month old Ret mice were collected from primary tumors mechanically dissociated in 150 μ l of phosphate-buffered saline (PBS). They were frozen until use. IL-17 was quantified by ELISA using R&D Systems. The concentrations in IFN- γ , IL-12, IL-10, TNF- α , KC, IL-1 β , and IL-6 were determined using a kit from Meso Scale Discovery according to supplier instruction. IL-1 β and IL-6 were also quantified from sera obtained following mandibular blood collection.

Single cell suspension procedures

Spleen were mechanically dissociated, homogenized, and passed through a 100 μ m cell strainer in PBS with 5% FCS and 0.5% EDTA. Primary tumors were mechanically dissociated and digested with 1 mg ml⁻¹ collagenase D and 0.1 mg ml⁻¹ DNase I for 20 min 37°C.

Cell staining and flow cytometry

Surface staining was performed by incubating cells on ice, for 20 min, with saturating concentrations of labeled Abs in PBS, 5% FCS and 0.5% EDTA. Mouse cell-staining reactions were preceded by a 15-min incubation with purified anti-CD16/32 antibodies (Abs) (Fc γ RII/III block; 2.4G2) obtained from hybridoma supernatants. Following anti-mouse Abs were used: FITC – conjugated anti-Ly6G (1A8), anti-B220 (RA3-632), PE – conjugated anti- δ TCR (GL3) and anti-NK1.1 (PK136), APC- conjugated anti-CD45.2 (104), anti-CD11b (M1/70), PerCP-Cy5.5 – conjugated anti-CD3 (145-2C11), anti- β TCR (H57-597), and anti-CD45.2 (104), Pacific Blue-conjugated anti-CD4 (RM4-5), V450-conjugated anti-Ly6C (AL-21), PE-Cy7-conjugated anti-CD11c (HL3), APC-H7-conjugated anti-CD8 (53–6.7). Abs were purchased from BD Biosciences except anti-CD11b, anti-B220 and anti- δ TCR from eBioscience. Data files were acquired on LSRII and analyzed using Diva software (BD Biosciences).

Microscopy

Hematopoietic cells were magnetically sorted from primary tumors with anti-CD45⁺ MicroBeads (Miltenyi Biotec). Then, CD45⁺ cells either from primary tumors or tumor draining LN were centrifuged onto a microscope slide using Cellspin 1 (Tharmac). Non-specific reactivity was performed with incubation of PBS, 1% BSA for 20 min at room temperature (RT). Biotin hamster anti-mouse $\gamma\delta$ TCR (GL3, BD Biosciences) and rabbit anti-mouse NOS2 (Calbiochem) used as primary Abs were incubated 1 hour at RT. Then, slides were washed in PBS, 1% BSA before being incubated for 30 min at RT with Alexa fluor 488-conjugated goat anti-rabbit (Jackson immuno research) and PE-conjugated streptavidine (BD Biosciences) used as secondary Abs. Finally nuclei were labeled with DAPI and slides were mounted in Vectashield mounting medium (Vector Labs). Images were acquired using an automated high-resolution scanning system (Lamina™, PerkinElmer) with 40X objective. Acquisitions were

performed with inForm for Lamina software (PerkinElmer) and images were analyzed with ImageJ and Panoramic Viewer (3DHISTECH).

In vivo treatments

For the neutralization of IL-1 β and IL-6 over a three month period, Ret mice received three times a week an i.p. injection of 50 μ g of anti-IL-1 β (Clone B122, BioXcell) and anti-IL-6 (Clone MP5-20F3, BioXcell) from week 1 to week 3, and then of 100 μ g of the same neutralizing antibodies from week 4 to week 12. Alternatively, 200 μ g of IL-1 receptor antagonist (Anakinra kindly provided by Pr. L. Mouthon, Cochin Hospital) was injected i.p. three times a week for three months. Diagnosis was performed once a week to detect the occurrence of primary tumor and cutaneous metastases. In the short-term treatment, 3 month old Ret mice were treated three times a week for 2 weeks with 100 μ g of anti-IL-1 β and anti-IL-6 antibodies.

Statistics

Comparison between incidence curves was performed using Wilcoxon test. Data are expressed as mean \pm SEM. The significance of differences between two series of results was assessed using the Mann-Whitney test. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). All statistical analyses were performed using Prism 6 software (GraphPad).

Study approval

Experiments were carried out in accordance with the guidelines of the French Veterinary Department and were approved by the Paris-Descartes Ethical Committee for Animal Experimentation (15–035, APAFIS #3646).

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was reported by the authors.

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Author’s contributions

L.D., L.B., R.L., and L.L. did experiments; L.D., and A.P.B. designed the study with the help of I.C., and L.D., and A.P.B. wrote the manuscript; and all authors critically reviewed the manuscript.

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